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Original Article

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Abstract

Porcine circovirus type 2 (PCV2) causes multisystemic diseases called porcine circovirus associated disease (PCVAD) in pigs leading to a significant economic loss in the swine industry. The objective of this study was to determine the efficacy of a developed sub-unit PCV2b vaccine using a recombinant truncated capsid protein expressed in *Escherichia coli* in PCVAD-affected pig farms. Five-week-old conventional pigs were allocated into 2 groups: vaccinated (n = 55) and control (n = 55) groups placed in the same building. The vaccinated pigs were given two shots of a developed vaccine with 2-week interval on day 0 and 14. Serum samples were collected from 10 randomly selected pigs of each group. IPMA and in-house indirect ELISA were used to evaluate antibody titers on 0, 14, 28, 77 and 119 days post first vaccination (dpv). Viral load of PCV2 in serum was determined by a modified real-time PCR on 0, 14, 28, 77 and 119 dpv. The IPMA results revealed that the vaccinated pigs had significantly higher antibody titers than the control pigs (4.90±0.99 log2, 2.50±0.85 log2, respectively) at 28 dpv ($p < 0.05$). While the ELISA test showed significantly higher differences ($p < 0.05$) of means S/P ratios between the two groups at 28 dpv (0.688±0.270 and 0.200±0.17) and at 119 dpv (1.440±0.38 and 0.808±0.47). The PCV2 viral loads in the vaccinated pigs were significantly lower than those of the control pigs at 28 dpv (4.97±0.25, 5.20±0.10), 77 dpv (4.71±0.38, 6.03±1.41) and 119 dpv (4.26±0.25, 5.03±0.67). Even though the vaccinated pigs showed lower viral loads than the control pigs, their viral loads were rather high from 28 dpv onward possibly due to natural PCV2 infection. The results indicated that vaccination by developed sub-unit PCV2b vaccine could induce humoral antibody and partially control PCV2 viral load in serum which resulted in higher protein concentration dose and/or using an appropriate adjuvant could enhance better efficacy of this developed sub-unit vaccine. This study demonstrated that the developed sub-unit PCV2b vaccine had potential for use as a future vaccine candidate. However, more field trials on production parameter and other parameters are needed for vaccine efficacy evaluation.

Keywords: antibody, capsid protein, PCV2b genotype, pig, sub-unit vaccine, viremia

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บทคัดย่อ

ซับยูนิตวัคซีนที่พัฒนาจากเชื้อเซอร์โคไวรัสชนิดที่ 2 บี สามารถกระตุ้นภูมิคุ้มกันที่จำเพาะในฟาร์มที่ประสบปัญหาโรคติดเชื้อเซอร์โคไวรัสชนิดที่ 2 ได้

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เชื้อเซอร์โคไวรัสชนิดที่ 2 ก่อปัญหาต่อสุขภาพของสุกรหลายระบบ มีอัตราการสูญเสียสูงและสร้างความเสียหายทางเศรษฐกิจต่อระบบการเลี้ยงสุกร การศึกษาครั้งนี้มีวัตถุประสงค์ เพื่อทดสอบประสิทธิภาพของซับยูนิตวัคซีนจากรีคอมบิแนนท์แคปซิดโปรตีนของเชื้อเซอร์โคไวรัสชนิดที่ 2 บี ซึ่งผลิตโดยระบบ *Escherichia coli* ในการกระตุ้นภูมิคุ้มกันชนิดสารน้ำและช่วยลดปริมาณของเชื้อเซอร์โคไวรัสชนิดที่ 2 ในกระแสเลือดของสุกรในฟาร์มที่ประสบปัญหาโรคติดเชื้อเซอร์โคไวรัสชนิดที่ 2 การทดลองแบ่งสุกรอายุ 5 สัปดาห์ออกเป็น 2 กลุ่ม โดยกลุ่มทดลองได้รับวัคซีน (n = 55 ตัว) และกลุ่มควบคุมได้รับเฉพาะสารสื่อวัคซีน (n = 55 ตัว) ทำการฉีดวัคซีน 2 ครั้งห่างกัน 2 สัปดาห์ (วันที่ 0 และ 14 ของการทดลอง) และทำการสุ่มเจาะเลือดจากสุกรทั้งสองกลุ่มจำนวนกลุ่มละ 10 ตัว เพื่อตรวจทางซีรัมวิทยาด้วยวิธีไอพีเอ็มเอและอีไลซาและตรวจหาปริมาณของไวรัสในซีรัมด้วยวิธีเรียลไทม์พีซีอาร์ในวันที่ 0 14 28 77 และ 119 วันหลังฉีดวัคซีนเข็มแรก จากผลการตรวจด้วยวิธีไอพีเอ็มเอ พบว่าสุกรในกลุ่มทดลองมีระดับไตเตอร์ที่สูงกว่ากลุ่มควบคุมอย่างมีนัยสำคัญที่ 28 วันหลังฉีดวัคซีนเข็มแรก (4.90±0.99, 2.50±0.85 log2) ในขณะที่ระดับภูมิคุ้มกันที่ได้จากการตรวจด้วยวิธีอีไลซานั้นมีความแตกต่างอย่างมีนัยสำคัญระหว่างกลุ่มทดลองกับกลุ่มควบคุมที่ 28 วัน (0.688±0.270, 0.200±0.17) และ 119 วัน (1.440±0.38, 0.808±0.47) หลังฉีดวัคซีนเข็มแรกตามลำดับ สำหรับปริมาณเชื้อไวรัสในกระแสเลือด พบว่าในสุกรทดลองมีปริมาณเชื้อไวรัสในกระแสเลือดต่ำกว่ากลุ่มควบคุมอย่างมีนัยสำคัญที่ 28 วัน (4.97±0.25, 5.20±0.10), 77 วัน (4.71±0.38, 6.03±1.41) และ 119 วัน (4.26±0.25, 5.03±0.67) หลังฉีดวัคซีนเข็มแรกตามลำดับ อย่างไรก็ตามแม้ว่าสุกรกลุ่มทดลองจะมีปริมาณของไวรัสในกระแสเลือดที่ต่ำกว่ากลุ่มควบคุม กลับพบว่าปริมาณของไวรัสในกระแสเลือดของกลุ่มทดลองนั้นมีแนวโน้มเพิ่มสูงขึ้นในช่วงวันที่ 28 หลังจากฉีดวัคซีนเข็มแรกเป็นต้นไป ซึ่งอาจเป็นผลเนื่องมาจากการติดเชื้อตามธรรมชาติภายในฟาร์มทดลอง จากผลดังกล่าวแสดงให้เห็นว่าซับยูนิตวัคซีนที่ผลิตขึ้นนี้สามารถกระตุ้นให้สุกรทดลองสร้างภูมิคุ้มกันชนิดสารน้ำที่จำเพาะได้ แต่สามารถควบคุมปริมาณของเชื้อเซอร์โคไวรัสชนิดที่ 2 ในกระแสเลือดได้เพียงบางส่วนเท่านั้น ซึ่งอาจเป็นผลเนื่องมาจากปริมาณของรีคอมบิแนนท์แคปซิดโปรตีนที่ใช้ในซับยูนิตวัคซีนนั้นยังไม่เพียงพอรวมไปถึงชนิดของสารสื่อที่ใช้นั้นอาจจะยังไม่เหมาะสม ซึ่งจำเป็นต้องทำการศึกษาเพิ่มเติมเพื่อปรับปรุงให้ซับยูนิตวัคซีนที่ผลิตขึ้นนี้มีประสิทธิภาพที่สูงขึ้น การศึกษาในครั้งนี้แสดงถึงศักยภาพว่าซับยูนิตวัคซีนที่ผลิตขึ้นนี้มีแนวโน้มที่จะพัฒนาต่อยอดในเชิงพาณิชย์ได้ อย่างไรก็ตามควรมีการศึกษาเพิ่มเติมในส่วนที่เกี่ยวข้องกับประสิทธิภาพในการผลิตสุกรในฟาร์มต่อไป

คำสำคัญ: แอนติบอดี แคปซิดโปรตีน เซอร์โคไวรัสชนิดที่ 2 บี สุกร ซับยูนิตวัคซีน เชื้อไวรัสในกระแสเลือด

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Introduction

Porcine circovirus type 2 (PCV2) has been identified as a causative agent of porcine circovirus associated disease (PCVAD) in pigs, an important swine disease having a huge impact on swine industry worldwide (Allan and Ellis, 2000; Chae, 2005). Currently, epidemiological studies indicate that PCV2b is a predominant genotype in the global pig population (Allan et al., 2007; Patterson and Opriessnig, 2010; Cortey et al., 2011) including Thailand (Jittimanee et al., 2011). The first available commercial PCV2 vaccine is an inactivated PCV2 oil adjuvant vaccine used for breeders (Reynaud et al., 2004). On the other hand, other three commercial vaccines are recommended for piglets. One is a killed chimera vaccine combining the antigenic capsid gene of PCV2 into PCV1 backbone (Fenaux et al., 2004). The others are subunit vaccines from a recombinant capsid protein expressed in baculovirus system (Fachinger et al., 2008). All commercially available vaccines are based on PCV2a genotype. Although previous experimental results indicated that current commercial vaccines based on PCV2a could confer cross protection against PCV2b in challenge models (Patterson et al., 2008; Fort et al., 2009; Opriessnig et al., 2010). Recently a PCV2 vaccine trial concurrent with PCV2, PRRSV and PPV infection mimicking field situation revealed that the PCV2b vaccine was more effective for reducing viremia than the PCV2a vaccine when challenged with only PCV2b or both PCV2a and PCV2b (Opriessnig et al., 2013). Additionally, novel vaccine prototypes based on PCV2b genotype such as sub-unit vaccines or modified live-attenuated vaccines are being developed and may provide better protection and lower vaccine costs. These developed

vaccines include recombinant capsid protein in different bacterial systems (Liu et al., 2001; Wang et al., 2008), viral-vectored vaccines (Wang et al., 2007; Fan et al., 2008[a]) and DNA vaccines (An et al., 2008; Shuai et al., 2013). Therefore, most of these studies were performed only in experimental models (mice or pigs). Moreover, it would be interesting to find protection efficacy of the PCV2 prototype vaccine under field condition.

Preliminary results revealed that a developed sub-unit PCV2b vaccine using a recombinant truncated capsid protein expressed in *Escherichia coli* could induce specific antibody responses in experimental pigs especially when vaccinated twice (Jittimanee et al., 2010). However, the efficacy of this vaccine trial under field condition is undergoing investigation. Therefore, the objective of this study was to investigate the efficacy of the developed sub-unit PCV2b vaccine in a PCV2-affected herd based on serology and viremic parameters.

Materials and Methods

Sub-unit vaccine preparation: A sub-unit PCV2b vaccine was prepared using a purified recombinant truncated Cap (rntCap) protein based on PCV2b genotype (GenBank accession No JQ866913) expressed in *E. coli* system as previously described (Jittimanee et al., 2012). The developed sub-unit PCV2b vaccine was mixed with a commercial water based adjuvant (MONTANIDE™ GEL-01 SEPPIC Inc, France) to make a final concentration of 50 µg/2ml/dose (the final concentration was based on preliminary results, data not shown).

Farms, housing and study designs: Field trial was conducted on a selected farm containing 1000 sows with multi-site production and all-in/all-out production system. The experimental farm was seropositive to porcine reproductive and respiratory syndrome virus (PRRSV) with no history of PRRS vaccination. This farm had continuous losses in recent months due to porcine circovirus associated diseases (PCVAD) without using PCV2 vaccination previously. Clinical signs were observed at approximately 12-18 weeks of age and preliminary PCV2 DNA was found in five serum pooled samples from 12, 15, 18, 21 and 24-week–old pigs by a routine PCR. One hundred and ten 5-week-old pigs were randomly allocated into 2 groups; a vaccinated group (n = 55) received two-shot vaccinations and a control group (n = 55) was received only an adjuvant. Both studied groups were in separated pens in the same building. Vaccination was done twice on day 0 and day 14 when the pigs were 5 and 7 weeks old. Serum samples were collected from 10 randomly selected pigs of each group before vaccination as the baseline and at 14, 28, 77 and 119 dpv for serological examination and quantitative PCR.

Serology:

Enzyme-Linked Immunosorbent Assay (ELISA): All serum samples were tested in duplication by an indirect rntCap-ELISA assay as described previously (Jittimanee et al., 2012). Briefly, 96-well flat-bottom plates (eBioscience Inc, CA, USA) were coated with the rntCap protein and blocked with 1% BSA in PBS. Subsequently, 100 µl/well of the 1:100 serum diluted were added and incubated at 37^0C for 30 min. Following 4 washes with phosphate buffer saline - 0.5% tween-20 (PBS-T), the final dilution of the horseradish peroxidase-labeled goat anti-swine IgG antibodies (Kirkegaard & Perry Lab, MD, USA) were added with further incubated at 37^0C for 30 min. The wells were again washed for 4 times and the reaction was developed using 100 µl of tetramethyl benzidine solution (Sigma-Aldrich, MO, USA). After incubation for 1 hour at room temperature in the dark, optical density was measured at 650 nm.

Immunoperoxidase monolayer assay (IPMA): IPMA was conducted for detecting the neutralizing PCV2 antibodies in pig sera. IPMA was performed in duplication as previously described (Liu et al., 2004) with some modifications. Briefly, 96-well flat-bottom plates containing the PK15/PCV2-infected cells and the negative control cells (mock-infected PK15 cells) were fixed in 4% formalin in PBS for 30 min at room temperature. Following 3 washes with PBS-T, 2-fold diluted-field serum samples and positive control (monoclonal mouse anti-PCV2 antibodies, Rural Technologies Inc, SD, USA) were added and then incubated at 37^0C for 1 hour. After 3 washes, 1 : 300 dilution of HRP-goat anti-swine IgG antibodies (New England Biolabs Inc, MA, USA) and 1 : 300 of HRP-conjugated rabbit anti-mouse Ig antibodies (Southern Biotech, AL, USA) were added. Following 1 hour of incubation at 37^0C, the plates were washed 3 times and 3-amino-9-ethyl-carbazol (Merck Millipore, MA, USA) solution was added and incubated for 30 min at 37^0C. Color developed from substrate was removed and then the plate was washed by tap water. The IPMA plate was examined for dark brown staining of PCV2-infected cells under an inverted light microscope.

Quantification of PCV2 DNA in serum samples: DNA extraction from serum samples (at 0, 14, 28, 77 and 119 dpv) was performed using NucleoSpin Extract Viral DNA Kit (Macherey-Nagel, Düren, Germany) for quantification of PCV2 genomic DNA copy numbers by a SYBR green real-time PCR as described previously (McIntosh et al., 2009) with some modification. Briefly, The PCR reaction was performed in a 25 µl volume containing 12.5 µl QuantiTect SYBR Green PCR Kit (QIAGEN, USA), 150 nM of each forward and reverse primer (F : 5′ATGCCCAGCAAGAAGAGTGGAAGAAG3′, R : 5′AGGTCACTCCGTTGTCCTTGAG ATC3′) and 3 µl of sample DNA extracts or 10-fold dilutions of standard PCV2 DNA, respectively. The amplification was performed under the conditions at 95^0C for 15 min followed by 40 cycles of 95^0C for 15 sec, 60^0C for 30 sec and 72^0C for 45 sec each. Means of viral loads were calculated from the individual viral load (PCV2 DNA genomic copies per ml of serum) of all analyzed animals of each respective sampling day.

Statistical analysis: Values of the IPMA titers and viral loads in serum were transformed to log2 and log10 values, respectively. Mean comparison of parameters (IPMA, ELISA and viral load) between the vaccinated and control groups was analyzed by unpaired *t*-test. The significance level (*p*-value) for all parameters was set at 0.05.

Results

Serology: IPMA and indirect ELISA were performed in duplication on total serum samples as the serological assays for this study. Mean log2 IPMA titers, prevalence of PCV2 seropositive pigs and mean group S/P ratios between the vaccinated and control groups of different sampling days were summarized in Table 1. The pigs vaccinated with a developed sub-unit PCV2b vaccine had better anti-PCV2 IgG response compared to the control pigs. The IPMA titers in the vaccinated pigs (mean titers: 4.90±0.99 log2) were significantly higher than those of the control pigs (mean titers: 2.50±0.85 log2) at 28 dpv ($p < 0.05$). However, at the time of PCV2 vaccinations (on day 0 and 14) and at 77 and 119 dpv, no significant differences of IPMA titers were detected between the two groups (Fig 1), but the ELISA results revealed significant differences ($p < 0.05$) in mean S/P ratios between the vaccinated and control groups at 28 dpv (0.688±0.270 and 0.200±0.17) and at 119 dpv (1.440±0.38 and 0.808±0.47) as shown in Figure 2. Moreover, the prevalence of ELISA seropositive pigs at 14, 28 and 77 dpv of the vaccinated group were 70% (7/10), 100% (10/10) and 80% (8/10), respectively, whereas only 30% (3/10), 20% (2/10) and 66.67% (6/9) of the control group were seropositive to PCV2 (Table 1).

Quantification of PCV2 DNA in serum samples: Quantification of PCV2 DNA in serum samples was performed in duplication using a modified SYBR green real-time PCR. The group means of log10 of PCV2 DNA genomic copies per ml of serum were calculated from the individual copy number of PCV2 genomic from each group transformed into log10 and summarized in Table 1. No statistical differences in means of PCV2 viral load in sera were observed between the vaccinated and control pigs at both vaccination days (day 0 and day 14). In contrast, means of PCV2 viral load in sera of the vaccinated group (viral load of 4.97±0.25, 4.71±0.38 and 4.26±0.25 log10) were significantly ($p < 0.05$) lower when compared to means of the control group (5.20±0.10, 6.03±1.41 and 5.03±0.67 log10) at 28, 77 and 119 dpv, respectively (Fig 3).

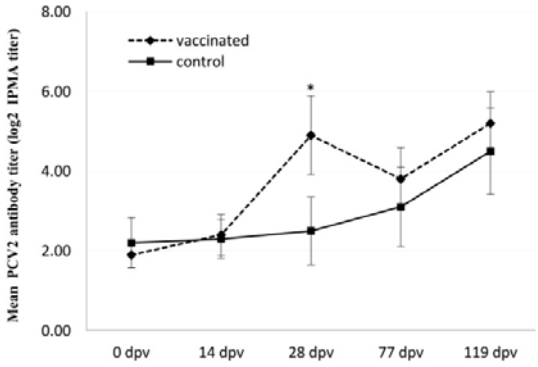

Figure 1 Mean group of log2 serum antibody titers measured by IPMA between vaccinated group (n = 10) and control group (n = 10) on different trial days (at 0, 14, 28, 77, and 119 dpv). Asterisks (*) indicate significant differences ($p < 0.05$). Error bars represent standard errors.

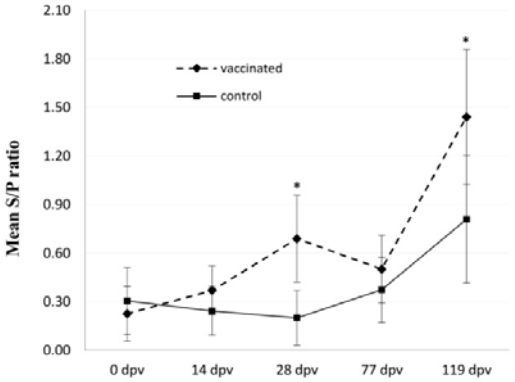

Figure 2 Mean group of sample-to-positive (S/P) ratios in serum samples obtained from vaccinated group (n = 10) and control group (n = 10) on different trial days (at 0, 14, 28, 77, and 119 dpv). An S/P ratio of 0.3 is considered positive. Asterisks (*) indicate significant differences ($p < 0.05$). Error bars represent standard errors.

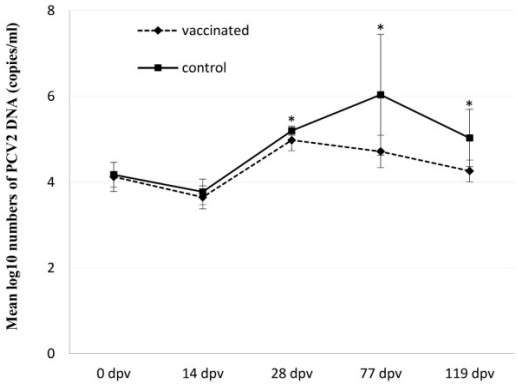

Figure 3 Mean group of log10 PCV2 DNA copies in serum samples obtained from vaccinated group (n = 10) and control group (n = 10) at 0, 14, 28, 77, and 119 dpv. Asterisks (*) indicate significant differences ($p < 0.05$). Error bars represent standard errors.

Discussion

Since PCV2 has been identified as a causative agent of PCVAD in pigs, the incidence of PCVAD in swine herds has become a major economic impact to swine industry worldwide (Allan et al., 1998; Allan and Ellis, 2000; Segalés et al., 2002). Currently, epidemiological studies indicate that PCV2b is a predominant genotype causing major losses in the global pig population (Patterson and Opriessnig, 2010; Cortey et al., 2011) including Thailand (Jittimanee et al., 2011). While several commercial PCV2 vaccines based on PCV2a are still widely used for the protection against PCVAD, cross protection against PCV2b has been demonstrated in experimental models (Patterson et al., 2008; Fort et al., 2009; Opriessnig et al., 2010). However, PCVAD outbreaks in vaccinated pig herds still occurred. Recently, a PCV2 vaccine trial concurrent with PCV2, PRRSV and PPV infections mimicking field situation revealed that the PCV2b vaccine was more effective for reducing viremia than the PCV2a vaccine when challenged with PCV2b or combined PCV2a/2b (Opriessnig et al., 2013). The result suggested that a new prototype vaccine based on PCV2b genotype might be useful and necessary.

Several previous studies of capsid protein expressed in *E. coli* suggested that this particular recombinant capsid protein had potential for use as a vaccine candidate (Zhou et al., 2005; Fan et al., 2008[b]; Marcekova et al., 2009). However, the efficacy of a sub-unit vaccine trial using a recombinant capsid protein is still needed. Moreover, it will be of interest to find protection efficacy of a sub-unit PCV2 vaccine prototype tested under field condition.

This study investigated the efficacy of the developed sub-unit PCV2 vaccine based on PCV2b recombinant capsid protein expressed in *E. coli* on enhancing humoral antibodies and reducing viremia in a PCV2-affected farm. The results from IPMA and indirect ELISA tests revealed that the pigs vaccinated with the developed sub-unit PCV2b vaccine had better anti-PCV2 IgG response compared to the control pigs. However, the significant differences between the vaccinated and control groups were observed only at 28 dpv by IPMA and at 28 dpv and 119 dpv by the indirect ELISA (Table 1). No significant differences between the two groups were shown at 14 and 77 dpv. This result indicated that the sub-unit PCV2b vaccine might induce a moderate level of IPMA titer (mean titer : 4.90±0.99 log2) and might partially control PCV2 infection measured by viremic reduction. Howeve, the PCV2 antibody seemed to rise again after the pigs were exposed to PCV2 naturally at 119 dpv. The short protection period of this developed sub-unit PCV2b vaccine might be due to insufficient recombinant capsid protein or inappropriate adjuvant type. The water based adjuvant in this established sub-unit vaccine was designed to improve the safety and efficacy of aqueous vaccines. It could stimulate humoral and/or cellular responses and also can be used with a wide range of antigenic media such as viral, bacteria and purified protein. However, this type of adjuvant may not induce long lasting immunity. Instead, using an emulsion oil adjuvant type may provide the long lasting immune response. Moreover, the percentages of seropositive pigs in the vaccinated group were higher than those of the control group at 14, 28 and 77 dpv.

The qPCR results revealed that means of PCV2 viral load in sera of the vaccinated group were significantly lower than those of the control group at 28, 77 and 119 dpv. These results indicated that the developed sub-unit PCV2b vaccine could reduce viral load in serum samples of naturally infected pigs. Nevertheless, the vaccinated group showed lower viral load than the control group. However, the viral load within the vaccinated group seemed to increase at 28 dpv onward possibly due to the natural infection. As a result, the sub-unit PCV2b vaccination could partially reduce the PCV2 viremia or improve the clinical signs caused by PCV2 infection.

In summary, the developed sub-unit PCV2b vaccine could enhance specific antibody and reduce the PCV2 viral load in serum samples in the studied PCV2-affected farm. Therefore, humoral immune responses induced by the developed sub-unit PCV2b vaccine could partially contribute to the PCV2 control and the clearance of PCV2 clinical signs under field conditions. In addition, increased concentration dose of protein used and/or using an appropriate adjuvant could enhance better efficacy of this developed sub-unit vaccine. This study demonstrated that the developed sub-unit PCV2b vaccine had potential for use as a future vaccine candidate. However, more field trials and other measured parameters such as production parameter, cell mediated immune response, viral shedding from excretion or organs are needed for better evaluation of vaccine efficacy.

Table 1 Results of IPMA, indirect ELISA assays and quantitative PCR. Gray shaded areas indicate PCV2 seropositive pigs from indirect ELISA test (S/P ratio>0.3).

Sampling days	IPMA		ELISA		qPCR	
	Mean log2 PCV2 titer ± sd		Prevalence (mean S/P ratio ± sd)		Mean log10 PCV2 (copies/ml ± sd)	
	Vaccinated	Control	Vaccinated	Control	Vaccinated	Control
0	1.90 ± 0.32	2.20 ± 0.63	2/10 (0.224 ± 0.17)	5/10 (0.303 ± 0.21)	4.12 ± 0.34	4.17 ± 0.29
14	2.40 ± 0.52	2.30 ± 0.48	7/10 (0.368 ± 0.15)	3/10 (0.242 ± 0.15)	3.64 ± 0.27	3.77 ± 0.30
28	4.90 ± 0.99*	2.50 ± 0.85	10/10 (0.688 ± 0.27)*	2/10 (0.200 ± 0.17)	4.97 ± 0.25*	5.20 ± 0.10
77	3.80 ± 0.79	3.10 ± 0.99	8/10 (0.500 ± 0.21)	6/9 (0.373 ± 0.20)	4.71 ± .0.38*	6.03 ±1.41
119	5.20 ± 0.79	4.50 ± 1.08	10/10 (1.440 ± 0.38)*	9/9 (0.808 ± 0.47)	4.26 ± 0.25*	5.03 ± 0.67

* Indicating significant ($p < 0.05$) differences between vaccinated and control groups

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